A TTX-sensitive inward sodium current contributes to spontaneous activity in newborn rabbit sino-atrial node cells

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1. Single cells were isolated from the sinus node region of rabbits (2 days old to adult) to study the age-dependent contribution of the sodium current (i_{Na}) to pacemaker activity.

2. Experiments were conducted in 50 mM Na^+–Ca^{2+}-free solution. All newborn cells (2–19 days) exhibited a TTX-sensitive, Mn^{2+}-insensitive fast inward Na^+ current (peak current density 115·5 ± 11·9 pA pF^{-1} at 0 mV). Fifty per cent of young cells (20–40 days) possessed the current, but only one in ten adult cells. Current density decreased with development independently of cell capacitance.

3. Newborn cells exhibited a noticeable window current. With development, the position of the activation curve was shifted in the positive direction, while the inactivation was unaltered, resulting in reduced overlap of the two curves and hence less window current.

4. In newborn cells, 3 μM TTX significantly reduced all measured parameters of spontaneous action potentials, slowing rate by 63%. In contrast, there was no significant effect of TTX on rate or most of the same paramenters in adult cells.

5. These results indicate that cells of the sinus node region exhibit a substantial TTX-sensitive current at birth. With development, both the density and frequency of occurrence of this current within the sinus node decrease, as does its contribution to automaticity.

Primary pacemaker activity in mammals is generated by the oscillatory properties of the sino-atrial node (SAN). Hallmarks of these nodal cells include the presence of the characteristic pacemaker (i_f) current (DiFrancesco, Ferroni, Mazzanti & Tromba, 1986), lack of the inward rectifier potassium current (i_{K1}) (Noma, Nakayama, Kurachi & Irisawa, 1984; Giles, van Ginneken & Shibata, 1986; Irisawa, 1987; Irisawa, Brown & Giles, 1993) and action potentials that are relatively insensitive to the fast Na^+ channel blocker tetrodotoxin (TTX) (Yamagishi & Sano, 1966; Kreitner, 1975; Kreitner, 1978; Kreitner, 1985). The presence of a sodium component has been debated in the primary pacemaker cells (Kreitner, 1975; Nathan, 1986; Irisawa *et al.* 1993), but even if present this component does not seem to be essential for automaticity (Yamagishi & Sano, 1966; Lenfant, Mironneau, Gargouil & Galand, 1968; Lipsius & Vassalle, 1978; Denyer & Brown, 1990; van Ginneken & Giles, 1991), based on studies both at the single cell level and in intact tissue. However, these conclusions are derived entirely from data in adult sinus node. To date there have been no single cell studies on the ionic current characteristics of SAN cells isolated from the newborn rabbit heart. Interestingly, Toda (1980) reported that the rate of rise in the intact sinus was greater at day 2 than at later ages, consistent with, as he suggested, there being a greater contribution of the sodium current (i_{Na}) in the newborn sinus node.

We have therefore addressed this question by studying sinus node cells isolated from newborn rabbits with the whole-cell patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Our results indicate the unexpected presence of a large TTX-sensitive sodium current, which appears to play an important physiological role in the young heart. In the presence of TTX, spontaneous rate is markedly reduced and most action potential parameters are affected.

METHODS

Isolation procedure

Protocols employed in these experiments were reviwed and approved by the Columbia University Institutional Animal Care and Use Committee.

Albino rabbits of three different age groups were chosen as the subject of these studies: newborn (N), 2–19 days; young (Y), 20–40 days; adult (A), > 40 days. Isolation of single SAN cells was performed following the methods described by DiFrancesco *et al.* (1986) with minor variations. Rabbits were anaesthetized by an

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I.M. injection of a mixture of xilazine (4·6 mg kg^{-1}; Fermenta Animal Health Co., Kansas City, MO, USA) and ketamine (60 mg kg^{-1}; Fort Dodge Laboratories Inc., Fort Dodge, IA, USA). The heart was removed and placed in normal Tyrode solution (mM: NaCl, 140; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 1; Hepes–NaOH, 5; glucose, 5·5), prewarmed to 37 °C and containing 0·5 ml heparin (1000 U ml^{-1}). The sinus node region was then carefully dissected free of surrounding tissue and cut into strips perpendicular to the crista terminalis. To test for possible cellular heterogeneity, in some experiments in newborn animals only small subregions from within the central area of the sino-atrial node were studied. Regardless of whether the entire central nodal area or a smaller subregion was dissected, and regardless of the location within the sino-atrial node of the subregion, the experimental results were comparable.

The dissociation procedure was similar to that described previously (DiFrancesco *et al.* 1986), with minor modification. In rabbits less than 15 days old we found the yield of viable single cells was higher if the enzyme concentrations were halved with respect to the adult dose. We did not observe any differences in the electrophysiological results in the newborn age range between the two (full or half) doses. In the adult preparations the cells studied were mainly of the elongated and spindle-like type. In general the newborn preparation yielded cells with the same shape, although some of them became shorter and wider during the calcium readaptation phase following isolation. No clear striations were detected. The same type of cell was always found, independent of the portion of the sinus region dissected. All the cells selected for this study were beating spontaneously in normal Tyrode solution at room temperature (20–22 °C) and all exhibited the pacemaker current (i_f).

Electrophysiological experiments

During the experiments aimed at isolating the sodium current, a low-Na^+, Ca^{2+}-free external solution was used which contained (mM): TEA-Cl, 90; NaCl, 50; CsCl, 5; Hepes, 10; $MgCl_2$, 0·5; glucose, 5·5; pH 7·4. When specified, TTX (Calbiochem) or $MnCl_2$ was added to the external solution at the desired concentration. In the same experiments the internal solution composition was (mM): aspartic acid, 80; CsOH, 75; Hepes, 10; EGTA, 10; NaCl, 10; $MgCl_2$, 1; CsCl, 30; ATP (disodium salt), 5; pH 7·2. Free Mg^{2+} concentration was calculated to be approximately 20 μM (Fabiato, 1988). In a few experiments a similar set of internal and external solutions, but with lower sodium, was used. In the external solution the Na^+ concentration was halved to 25 mM and in the internal solution the ATP(Na_2) was substituted with ATP(Mg). The perfusion system allowed a complete change of the solution surrounding the cells in less than 3 s (DiFrancesco *et al.* 1986). In current-clamp experiments the external superfusing solution was normal Tyrode solution, while the intracellular solution contained (mM): aspartic acid, 130; KOH, 146; NaCl, 10; Hepes, 10; EGTA, 5; $CaCl_2$, 2 (pCa 7); ATP(Mg), 2. All experiments were conducted at room temperature with the sole exception of the data illustrated in Fig. 1.

Pipettes were pulled from borosilicate glass capillaries and, when filled, had a resistance of 1–4 MΩ. On-line capacitance correction and series resistance compensation (40–80%) were employed. The value of the current was calculated as the difference between peak and completely inactivated current (30 ms after the onset of the pulse). The voltage values were not corrected for the junction potential which, under our conditions, was experimentally determined (Barry & Lynch, 1991; Neher, 1992) to be approximately 10 mV. For the sodium current all traces recorded were low pass filtered at a frequency of 10 kHz and the sampling rate was set at 20 μs. pCLAMP 5·5 and 6·0 software were used for acquisition and analysis of voltage-clamp data, and Axopatch 2·0 software for current-clamp experiments.

Statistical analysis

Data are expressed as means ± S.E.M. Differences were determined using the Student's group t test, paired t test or nested ANOVA as indicated in the text. Differences were considered significant if $P < 0·05$.

RESULTS

The characteristics of adult pacemaker cells have been described earlier by several authors. After enzymatic dissociation it is possible to obtain cells of different shape (Belardinelli, Giles & West, 1988; Denyer & Brown, 1990; van Ginneken & Giles, 1991). However, certain features are peculiar to all sino-atrial cells: presence of the pacemaker current (i_f), absence of the inward rectifier potassium current (i_{K1}) and presence of spontaneous activity in normal Tyrode solution. These features, along with the generally elongated shape and absence of observable striations, were assumed as guidelines in the investigation of newborn SAN cells.

Figure 1 shows typical records of i_f in an adult (*A*) and a newborn (*B*) cell. The external solution contained Mn^{2+} (2 mM) and Ba^{2+} (1 mM) to block calcium and potassium currents. As expected, both cells responded to a hyperpolarizing step with a slowly activating inward current. However, the tail currents recorded at the onset of the return to the holding potential were strikingly different. In the adult the tail simply reflects the deactivation of i_f at −35 mV, whereas in the newborn a fast inward current is activated (arrow). Due to the presence in the solution of L-type calcium channel blocker (Mn^{2+}, 2 mM), this current was not likely to be carried by calcium ions through the L-type channels. The ionic nature of the current present in the newborn cells was investigated in subsequent experiments.

An external solution lacking Ca^{2+} and having one third of the normal Na^+ was employed in these later experiments to reduce the size of the inward current, improve voltage control and eliminate the calcium current. In Fig. 2 a newborn cell was clamped for 2 s at −80 mV and then to 0 mV for 30 ms. A rapidly inactivating inward current was activated. Since we could not exclude the possibility that sodium ions, in the absence of calcium, could permeate through calcium channels and contribute to the total current, we first tested the effect of Mn^{2+} (panel *A*). In this and in three more cells, Mn^{2+} (2 mM) did not reduce the inward current. Panel *B* illustrates that TTX (3 μM) completely abolished the current in this cell. In a total of nine cells the same concentration of TTX either markedly reduced or fully blocked the current (mean reduction, 95·0 ± 2·0% from a control current density of 110·5 ± 15·5 pA pF^{-1}). Thus, the rapidly inactivating inward current present in newborn cells isolated from the

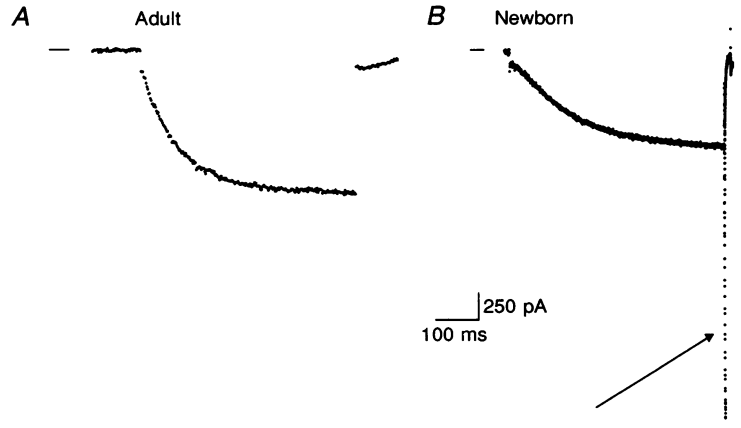

Figure 1. Pacemaker (i_f) current in adult and newborn SAN cells

A, a typical i_f current activates on hyperpolarization to −125 mV in an adult SAN cell. The very negative potential was employed to confirm that, even at extreme holding potentials, a fast inward current could not be generated upon depolarization back to the holding potential of −35 mV. *B*, a similar current recorded in a newborn cell with a step to −90 mV. Note that an inward tail current activated during return to the holding potential (−35 mV) is present in the newborn (arrow) but not in the adult myocyte. Traces recorded at 35 °C in normal Tyrode solution containing Ba^{2+} (1 mM) and Mn^{2+} (2 mM).

sinus node region is carried through TTX-sensitive sodium channels. Although not all cells from the young group exhibited the rapidly inactivating sodium current (see Fig. 3), those that did possessed similar TTX sensitivity (94·0 ± 4·3% reduction by 3 μM TTX, from a control current density of 32·0 ± 16·6 pA pF^{-1}, $n = 5$) and Mn^{2+} insensitivity (no reduction by 2 mM Mn^{2+}, $n = 2$). The presence of a TTX-sensitive current was confirmed further in five newborn cells under physiological calcium concentrations along with either Mn^{2+} (2 mM) and Ni^{2+} (100 μM) or Mn^{2+} (2 mM) and nifedipine (10 μM), (data not shown).

All newborn cells studied possessed the sodium current shown in Fig. 2, as well as the pacemaker current i_f. We characterized these newborn cells further by testing for the presence of the inward rectifier potassium current (i_{K1}), defined as a 2 mM Ba^{2+}-sensitive current. In four cells studied, no evidence of i_{K1} was detected (data not shown). This current is absent in the adult node, but is a major component in adult atrial cells (Giles & Imaizumi, 1988). The lack of i_{K1} agrees with the conclusion that the cells under study were SAN pacemaker cells.

The age-dependent expression of the TTX-sensitive current was then analysed. All cells studied in the range 2–19 days exhibited the sodium component ($n = 24$), whereas in the 20–40 day range it was detected in 50% of the cells (9 of 18). In the adult group only one cell out of ten showed the current. In Fig. 3, newborn and young groups were divided into a total of four subgroups: 2–9, 10–19, 20–29 and 30–40 days of age. In panel *A* the fraction of cells exhibiting the current is plotted. One might argue that the decrease in incidence with age (from 100% to 50% to

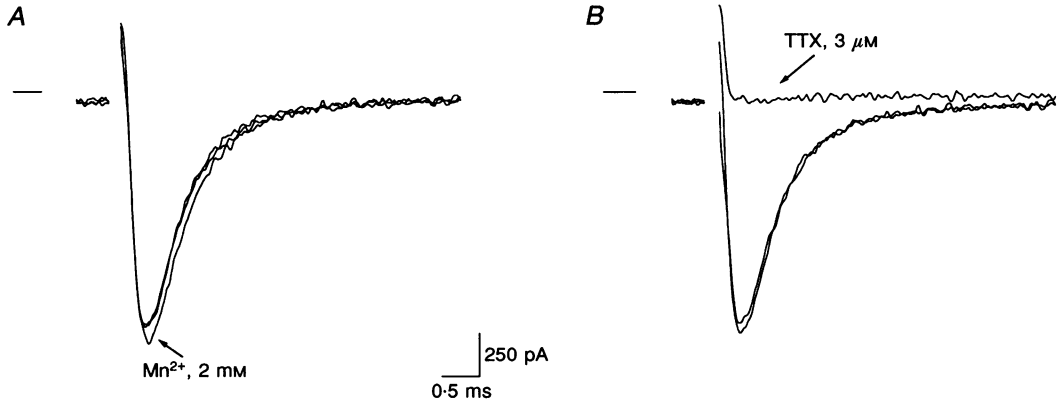

Figure 2. Effect of Mn^{2+} and TTX on the sodium current

Superimposition of current records before, during and after addition of the blocking agent. The lack of effect of Mn^{2+} (2 mM) (*A*) and full current block by TTX (3 μM) (*B*) are apparent. Voltage step was from −80 to 0 mV. Capacitative transients during the first 140 μs after the onset of the pulse have been blanked.

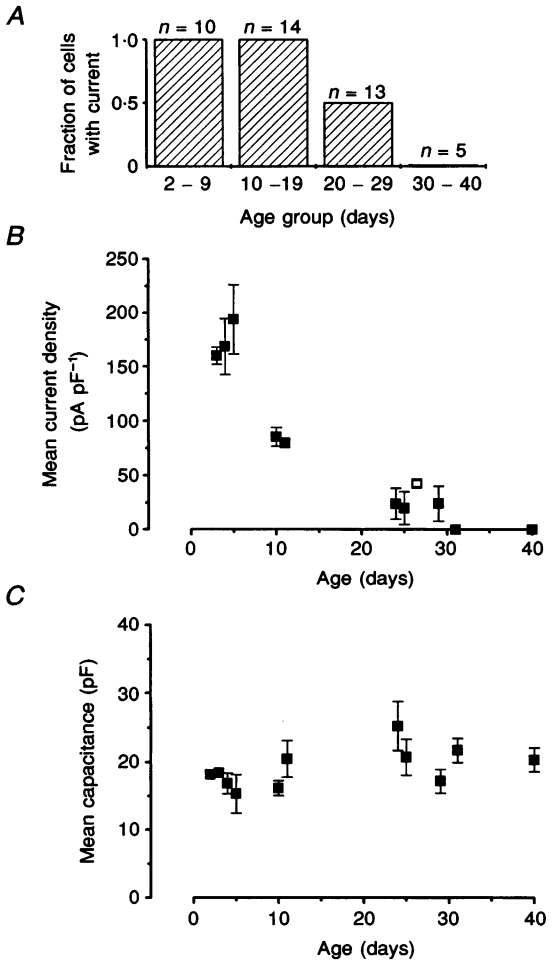

Figure 3. Decrease in sodium current with postnatal development

A, bar graph showing the fraction of cells possessing the TTX-sensitive sodium current in 4 different age groups. *B*, mean density at different ages showing a progressive decrease. Since the 20–29 day group included both cells with and without the current, we calculated separately the mean current density of only those cells from this time period that demonstrated the current (□), to confirm the decrease in density. *C*, age dependence of average cell capacity, showing no significant change with age.

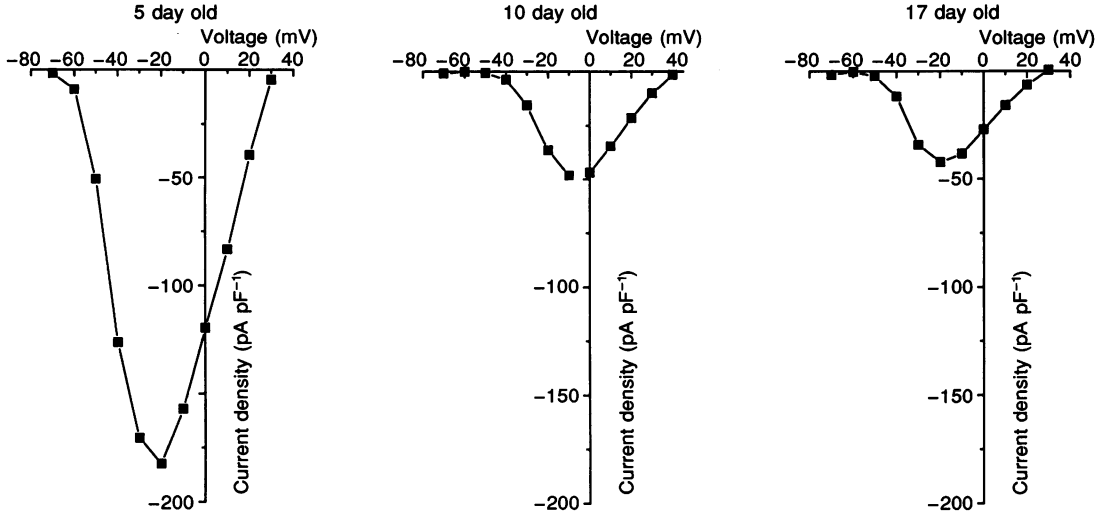

Figure 4. Current–voltage relations of representative cells, plotted at three different stages of postnatal development

The current density scale is equal for all curves to emphasize the progressive decrease of the current. External and internal sodium concentrations used in these measurements were 25 and 10 mM, respectively. A total of 16 *I–V* curves were obtained over the range 3–29 days, and no age-dependent shift in either the position of the peak or the reversal potential was observed.

nearly 0%) was consistent with two cell populations with distinct but constant characteristics, and only the proportion changed with age. The data in Fig. 3*B* argue against this interpretation. In this case the density was calculated using the current elicited upon stepping to 0 mV, normalized for cell capacitance. It is apparent that there is a marked age-dependent decrease in density as early as day 10, when all cells continued to exhibit the current. Further, the reduced density was not associated with an increasing cell size, since the average capacitance did not change significantly within the time frame studied (Fig. 3*C*).

Since current density was measured for a fixed step to 0 mV, we next determined whether there was any age-dependent shift of the *I–V* curves along the voltage axis that might underlie the decrease in current density. Figure 4 shows typical *I–V* relations for three different ages (5, 10 and 17 days). In a total of sixteen cells from animals between 3 and 29 days old, there was no significant age-dependent correlation in either the position of the peak of the *I–V* relation or in the reversal potential. However, we did observe a slightly more negative activation threshold in the early newborn cells. This was studied further by measuring the full activation and inactivation relations at different ages.

Figure 5 shows mean activation and inactivation curves obtained in the newborn and young group. While the inactivation curve did not vary appreciably with development, the position of the activation curve was shifted

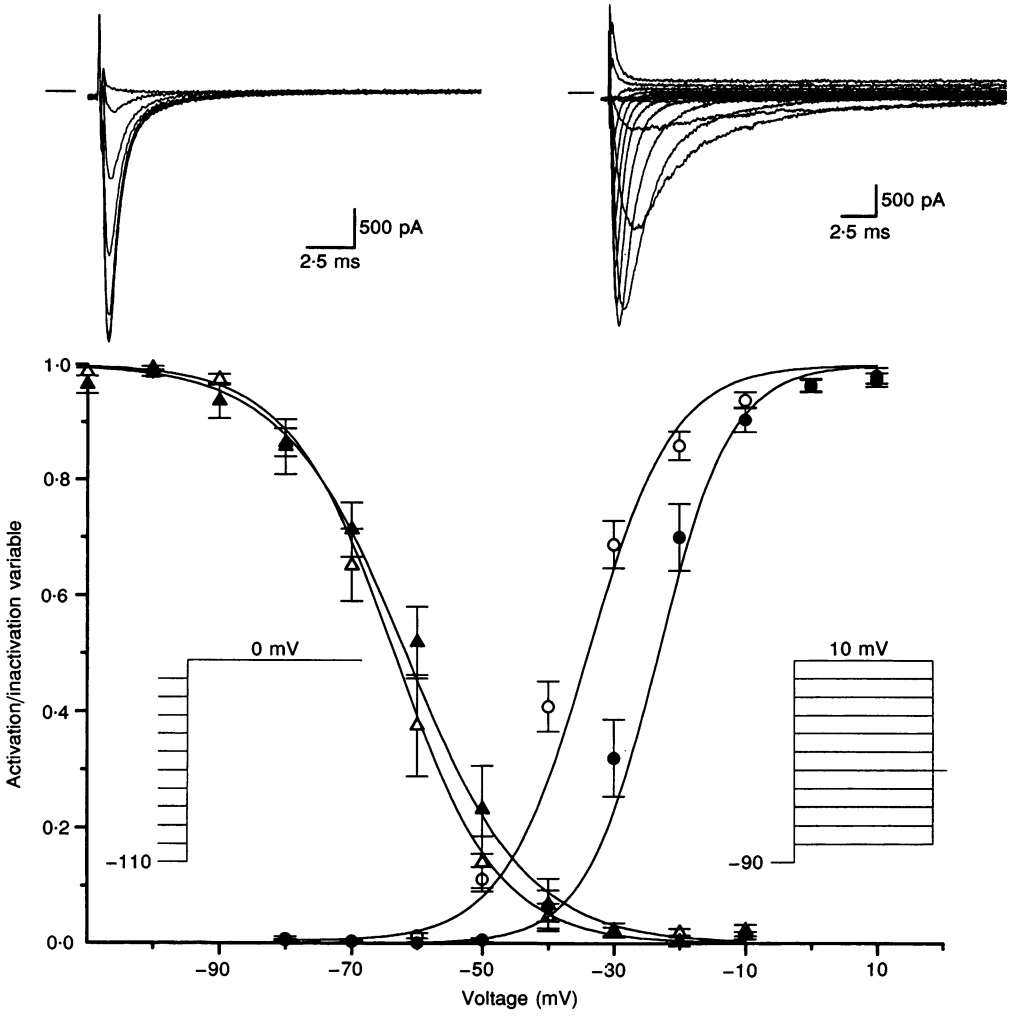

Figure 5. Activation and inactivation relations in two different age groups

Upper panels depict a family of current traces generated during inactivation (left) and activation (right) protocols in two different newborn cells. Average activation ($n = 7$) and inactivation curves ($n = 6$) for the newborn (○, △) and young (●, ▲) ($n = 5$ and 8, respectively) cells are plotted in the lower panel. Insets illustrate the two voltage protocols. Mid-points of inactivation curves were $-63{\cdot}5 \pm 1{\cdot}0$ and $-61{\cdot}7 \pm 1{\cdot}1$ mV; and of activation curves were $-33{\cdot}9 \pm 0{\cdot}7$ and $-23{\cdot}2 \pm 0{\cdot}8$ mV, respectively for newborn and young cells. To give smooth curves, the data were fitted according to the Boltzman equation: ($Y = 1/(1 + \exp(E - E_h)/s)$ for inactivation and $Y = 1/(1 + \exp(-(E - E_h)/s))$ for activation where s is the inverse slope factor, and E_h is the half-activation (or inactivation) voltage. The two activation curves differed significantly by nested ANOVA.

Table 1. Effect of TTX on spontaneous action potential characteristics

	Frequency (beats min^{-1})	MDP (mV)	Phase 0 (V/S)	Phase 4 (V/S)	Overshoot (mV)	Threshold (mV)
Newborn *						
Control	90·2 ± 8·5	−55·2 ± 4·0	14·3 ± 4·7	0·035 ± 0·004	50·9 ± 4·2	−41·2 ± 4·7
TTX (3 μM)	33·3 ± 8·0†	−52·4 ± 4·6†	5·1 ± 2·2†	0·015 ± 0·005†	43·0 ± 4·4†	−35·0 ± 5·2†
Adult						
Control	76·4 ± 17·2	−58·0 ± 1·9	2·6 ± 0·8‡	0·043 ± 0·011	32·8 ± 4·1‡	−42·3 ± 2·7
TTX (3 μM)	68·3 ± 15·4§	−59·2 ± 1·6	2·2 ± 0·8	0·025 ± 0·006	32·4 ± 4·1	−45·1 ± 2·3†

Values are means ± S.E.M. * 1 cell stopped in TTX; data are values from last 3 action potentials before cessation of spontaneous activity; a sixth cell stopped abruptly upon exposure to TTX and was discarded. † $P < 0·05$ relative to control by paired t test; ‡ $P < 0·05$ relative to newborn control category by group t test; § $P < 0·1$ relative to newborn TTX category by group t test. $n = 5$ in all cases.

toward more depolarized voltages. When curves were best fitted by a Boltzman function (see figure legend), the mid-point values of activation and inactivation were, respectively: −33·9 ± 0·7 and −63·5 ± 1·0 mV for newborn, and −23·2 ± 0·8 and −61·7 ± 1·1 mV for young. In the newborn a substantial window component is hence predicted in the range −60 to −30 mV under these experimental conditions, which becomes smaller with age due to the positive shift of the activation curve. However, since the solution used did not contain calcium, a positive shift of all curves, and the associated window current is expected to occur when recording in normal Tyrode solution.

To assess the possible contribution of a TTX-dependent window current to diastolic depolarization under physiological conditions, we investigated the effect of TTX on spontaneous action potentials. In Fig. 6, spontaneous action potentials recorded from a representative newborn SAN cell are shown. A marked slowing in rate was observed with 3 μM TTX (Fig. 6*B*). Quantitative analysis of the action potential parameters from five such experiments are shown in Table 1. All parameters studied were significantly changed in the presence of TTX. While some of these changes such as slope of phase 0 (maximum upstroke velocity), overshoot and threshold, can easily be

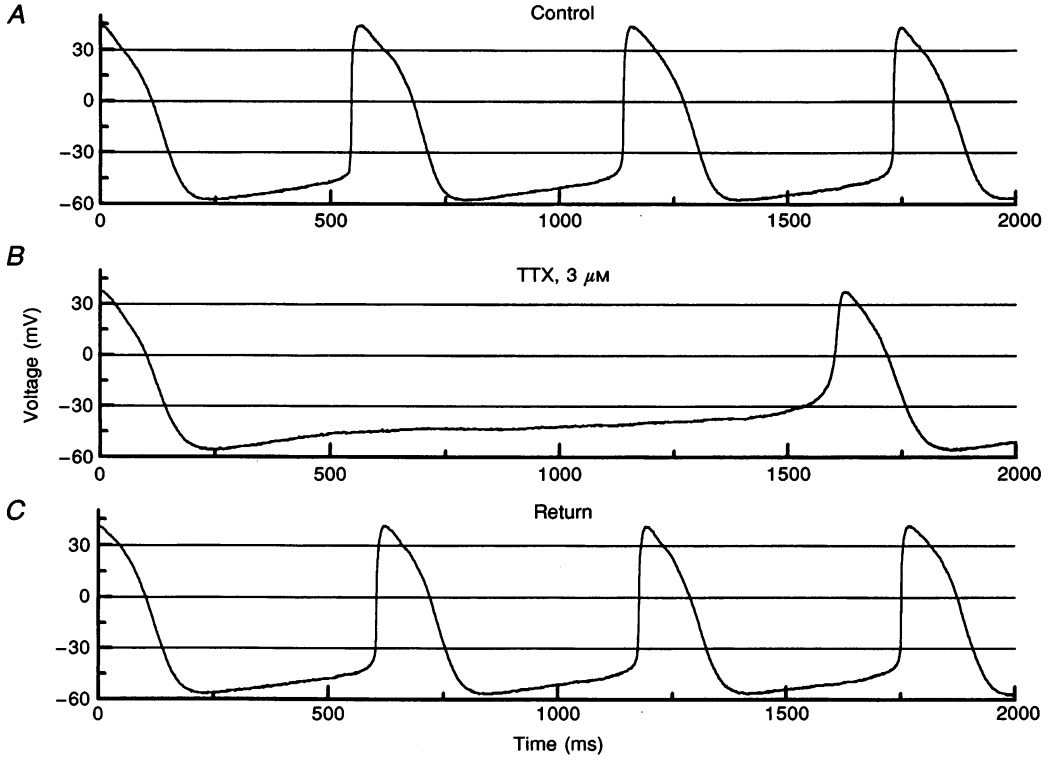

Figure 6. Effect of TTX on action potentials recorded in a newborn SAN single cell

A and *C*, control and return recordings of spontaneous activity. *B*, same but in the presence of TTX (3 μM).

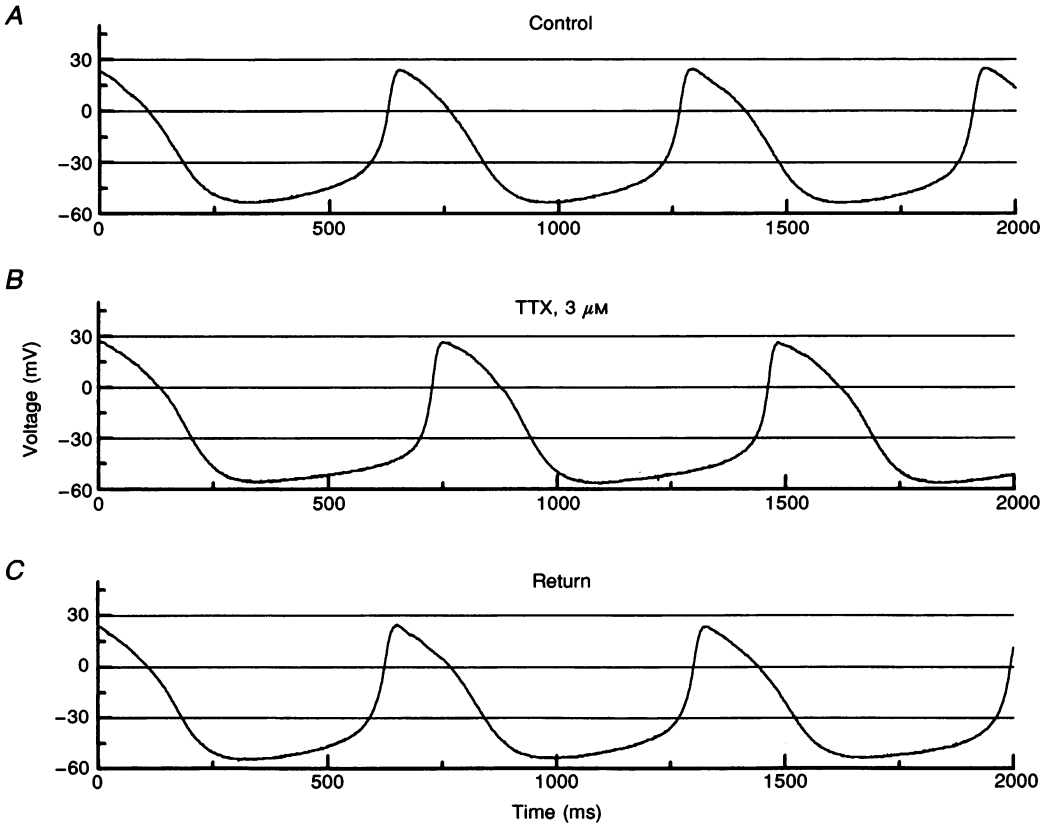

Figure 7. Effect of TTX on action potentials recorded in an adult SAN single cell
A and *C*, control and return recordings of spontaneous activity. *B*, same but in the presence of TTX (3 μM).

explained by assuming that i_{Na} contributes to the fast upstroke, others, such as maximum diastolic potential (MDP) rate, and slope of phase 4 (slow diastolic depolarization rate), suggest that a TTX-sensitive component may affect, either directly or indirectly, diastolic depolarization. Figure 7 shows an experiment similar to that of Fig. 6, conducted on an adult cell. The effect of TTX (3 μM) on action potential parameters are again summarized in Table 1 from five such cells. In the presence of TTX no significant change was detected, other than a slight negative shift of the threshold. There was a modest slowing of rate and reduction of phase 4, but these effects did not reach statistical significance.

Consistent with a contribution of i_{Na} to the newborn but not to the adult SAN action potential, phase 0 and overshoot differed significantly between the two control groups (Table 1). After TTX (3 μM) treatment, these parameters no longer differed between newborn and adult. However, a possible difference in spontaneous rate was revealed ($P < 0{\cdot}1$), with the newborn cells exhibiting a markedly slower rate than the adult cells in the presence of TTX.

DISCUSSION

Our data show that a fast inward sodium current is normally present in newborn rabbit SAN cells, and slowly disappears during the first 40 days of postnatal life. The possible role of a sodium current in the early stage of postnatal SAN development was first proposed by Toda (1980), based on differences in the action potential shape in newborn and mature SAN tissue. Other authors have shown, in different species, that the intact heart rate is faster in newborn than in adult (Toda, 1980; see also Macfarlane & Veitch Lawrie, 1989, for multiple examples). The presence of a TTX-sensitive i_{Na} current, such as that reported here, can at least partially account for these early observations.

The first evidence of a fast inward current at the single cell level, in our experiments, occurred during return to the holding potential of −35 mV from a negative step to activate the hyperpolarizing activated current, i_f. The correct description of this tail in terms of a sodium conductance was accomplished using ion substitution and pharmacological dissection. TTX sensitivity, fast kinetics,

and lack of action of Mn^{2+} (2 mM) were used as criteria to identify this as a fast Na^+ current. One might be concerned that in the absence of external calcium, sodium ions could permeate calcium channels. However, the absence of calcium chelator agents in the external solutions should keep the Ca^{2+}/Na^+ permeability ratio of calcium channels sufficiently high to inhibit Na^+ flux (Almers & McCleskey, 1984; Hille, 1992). Indeed no effect was evident during Mn^{2+} (2 mM) superfusion.

Since we detected a similar current in only 1 of 10 adult cells it was interesting to study the presence of this current during development. The findings that during development progressively fewer cells express this current, and that a clear age-dependent decrease in current density exists (in the absence of any significant increase in cell capacitance), provide strong arguments in favour of a developmental process in the nodal cell population. If, in fact, we were dealing with two populations, one with i_{Na} in the newborn, and one without the current in the adult, the average density of the current in that subset of cells exhibiting i_{Na} would not be expected to change. In addition, if the i_{Na}-containing cells in the newborn represented a 'contaminating' peri-nodal population, we would have expected to observe a difference in the expression of this current as we studied different subregions of the nodal area (see Methods). Since we did not observe cellular heterogeneity in different SAN subregions, it appears that a TTX-sensitive Na^+ current is widely present throughout the central region of the newborn node. Thus, from the data in Fig. 3 it is clear that in the early stage of postnatal life a large number of Na^+ channels are expressed (average current density $115{\cdot}5 \pm 11{\cdot}9$ pA pF^{-1} in 50 mM external Na^+ for the 1–20 day range), and that these channels slowly disappear or cease to function.

It is worth considering the possible reasons for the loss of current density with age. Since in general we used higher enzyme concentrations in the older animals, one might consider the results to be simply an artifact of the isolation, with the higher enzyme concentration leading to loss of Na^+ current. This clearly is not the case. First, density is falling by day 10, yet the enzyme concentrations remained low until day 15. Second, in our earliest experiments we used the higher concentration even with the youngest age, with no difference in electrophysiological results. Finally, the absence of the current in the adult, where the higher enzyme concentrations were used, is entirely consistent with all earlier intact tissue data from the adult sinus. We can also dismiss, as the sole explanation for the loss of current with age, a progressive positive shift of the curve to voltages outside the physiological range. We know this is not the case because in some adult cells we stepped as positive as +50 mV without unmasking a hidden current. Further, there is a clear age-dependent decrease in current density, independent of cell size, before the current totally vanishes. Another possibility is that the developmental fall in density results from the turning off of the expression of a neonatal Na^+ channel and the new expression of a more mature one, with different voltage characteristics and a lower single channel conductance. This possibility cannot be entirely eliminated without conducting single channel experiments, but it is insufficient by itself to account for our data. We do not observe simply a reduction in current density but the eventual loss of all detectable Na^+ current. Thus, one would have to postulate that expression of this second type of Na^+ channel also stops at a certain age. Also arguing against the idea of two distinct Na^+ channels is the observation that both the newborn and young cells show the same sensitivity to TTX and Mn^{2+}, despite somewhat different voltage dependence. Finally, this age-dependent shift in activation voltage is itself worth considering. A developmental shift in the inactivation voltage dependence of ventricular Na^+ channels has already been reported (Zhang, Robinson & Siegelbaum, 1992). Further, acute differences in voltage characteristics of i_{Na} have been observed upon exposure to β-adrenergic agonists (Ono, Fozzard & Hanck, 1993), and have been suggested to occur as a result of different modes of the i_{Na} channel (Bohle & Benndorf, 1995).

The presence of a slow diastolic depolarization, the relatively small dV/dt during the upstroke and a smooth transition between these two phases are, among others, specific properties of the sinus node action potential in the adult primary pacemaker cells. In our experiments, TTX (3 μM), when superfused on a spontaneously beating adult cell, did not modify significantly the majority of the action potential parameters (Table 1), in agreement with the lack of TTX-sensitive current observed in voltage-clamp measurements. The presence, at the single cell level, of the sodium current in the adult rabbit sinus node is still controversial (Nathan, 1986; Denyer & Brown, 1990; Irisawa *et al.* 1993). Certainly its contribution to the action potential shape is not as pronounced as it appears to be in the newborn (Fig. 6). Indeed, the spontaneous activity in the newborn is strongly affected by the TTX-sensitive sodium current. Cells isolated from border regions of the adult sino-atrial node, often called transitional or secondary pacemaker cells, show faster slope of phase 0 and a more definite transition between phases 4 and 0 (Lipsius & Vassalle, 1978; Nathan, 1986; Denyer & Brown, 1990). Action potentials recorded from the central zone of the sino-atrial node in newborn (Fig. 6) showed characteristics similar to the adult transitional type. However, we can exclude the possibility that we were selectively recording from cells from peripheral regions because of the criteria adopted in the dissection. Furthermore, it is expected that cells with large i_{Na} would have precisely these characteristics. Finally, the decrease in the upstroke velocity and in the overshoot during TTX superfusion indicate that Na^+ channels are activated when the newborn nodal cell depolarizes spontaneously. This view is also confirmed by the positive shift of the threshold

during TTX superfusion, since when sodium channels are blocked, a more positive potential must be reached to activate L-type calcium currents.

The effect of TTX on the slope of the pacemaker depolarization is more difficult to explain on the basis of the i_{Na} kinetic properties. The TTX-induced slowing of phase 4 could, however, be directly dependent on the presence of a TTX-sensitive window current at diastolic potentials. Determination of the position of the activation and inactivation curves indicates that in the newborn this is a possibility, since a substantial overlapping of the two curves is present. The 'window' current range in newborn spans the pacemaker region (under our recording conditions), making it possible that a steady-state contribution of the sodium current exists. The density of current necessary to generate the pacemaker depolarization is very small (DiFrancesco, 1991), and the contribution of a window current could thus be significant. The positive shift of the activation curve observed in the young population decreases the amplitude of the window current and its potential contribution to the pacemaker potential range.

Alternatively, the effect of TTX on phase 4 could result from an indirect mechanism secondary to effects of TTX on earlier portions of the action potential. For example, the somewhat reduced overshoot may decrease i_K activation, in turn resulting in a reduced MDP (see Table 1). This can lead to a lesser activation of the i_f current in the presence of TTX relative to control, and thus to a slowing of rate.

Consistent with our single cell results, several reports have indicated lack of TTX dependence of rate in the adult intact sinus node (Yamagishi & Sano, 1966; Lenfant *et al.* 1968; Kreitner, 1975; Lipsius & Vassalle, 1978). Based on the results at the single cell level we conducted a few pilot extracellular recordings ($n = 3$) on intact newborn sinus nodes to evaluate the effect of 10 μM TTX on the frequency of contraction. In each case, we observed a reversible increase in cycle length ($43 \pm 25\%$). Although these data are preliminary, and only a complete mapping of the newborn sino-atrial node with intracellular microelectrodes would fully address the issue of TTX sensitivity in the intact tissue, these experiments are consistent with the conclusion that a significant population of cells with a TTX-sensitive sodium current is present in the newborn sino-atrial node, and that indeed this sodium current makes a physiological contribution at this age.

In conclusion, our data suggest that the presence of a sodium current is physiologically relevant to the rate determination in the newborn sino-atrial node, and that with development its contribution progressively vanishes. The precise level of the contribution of this current to pacemaker depolarization at various stages of development remains to be determined. In addition, in TTX-treated cells we observed a persisting difference in spontaneous rate between newborn and adult cells. This raises the possibility that there are other age-dependent differences in the ionic basis of the SAN action potential that await elucidation and which may serve to compensate for the developmental reduction of i_{Na}.

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